SOME EFFECTS OF METHYL CHOLANTHRENE ON THE MORPHOLOGY AND GROWTH OF YEASTS

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With increasing knowledge of the chemistry of cholanthrene and related compounds and their action on the cells of mammals, it has seemed desirable to study their effects on organisms with less complex structures and interrelations of parts, in order to determine the action of these compounds on the morphology of individual cells and on the relation of cells to each other in a relatively simple group of plants such as yeasts. Some growth and fermentation studies have been undertaken, but much more data must be secured by the use of more refined methods before any broad generalizations can be safely made. As this manuscript was nearing completion, Goldstein[1] reported that 1,2,5,6 dibenzanthracene and methyl cholanthrene increased cell division in a bacterium, *Escherichia communior*, so that approximately 50 per cent more cells were present in the eighth to ninth hour than in the control, while phenanthrene showed no effect. No details of methods are given beyond the suggestion that the carcinogens were present in colloidal suspensions.

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[1] Goldstein, Samuel. 1937. A microbiological test for carcinogenic hydrocarbons. Science N.S. **86**: 176, 177.

Six organisms from our collection of pathogenic and saprophytic fungi were utilized in the preliminary work: *Zymonema dermatitidis* (Gilchrist & Stokes) Dodge, isolated from a case of blastomycotic dermatitis and reported by Moore[2] and since grown on malt extract agar; *Mycocandida onychophila* (Pollacci & Nannizzi) Langeron & Talice, received in 1934 from the Centraalbureau voor Schimmelcultures, Baarn; *Castellania tropicalis* (Castellani) Dodge, originally from Castellani, received in 1927 from Bailey K. Ashford through the kindness of Willard C. Greene; *Saccharomyces ellipsoideus* Hansen, a champagne strain received in 1936 from the American Wine Company through the kindness of Adolf Heck, Jr., and a Tokay strain received in 1935 from P. L. Varney of the Bacteriology Department of the Washington University Medical School; and finally some observations were made upon a very pleomorphic organism isolated by Gruner from the blood of a patient with carcinoma, and given me in 1936 by R. R. Rife.

METHODS

For studies of morphology, colonies on Sabouraud glucose agar (using Bacto products of the Digestive Ferments Company) were imbedded in celloidin, using the technique developed in this laboratory and outlined by Moore,[3] and stained with iron haematoxylin.

Liquid cultures were centrifuged, most of the supernatant liquid decanted, and Hermann's fixative added. After twelve hours, the material was again centrifuged and the fixative decanted. The material was washed several times with distilled water to remove the fixative, centrifuged, and mounted in a drop of Maneval's stain in Amann's lactophenol solution. The repeated centrifuging may have dissociated the cell groups slightly, the amount depending upon the thickness of the geli-

[2] Moore, Morris. 1933. Blastomycosis: report of a case, with a study of an etiologic factor and a classification of the organism. Ann. Mo. Bot. Gard. **20**: 79–118, *pl. 6, 7.*

[3] Moore, Morris. Microscopy. In Dodge C. W. 1935. Medical Mycology: fungous diseases of man and other mammals. pp. 71–73.

fied sheaths of the cells and of protoplasmic connections persisting between the cells. However, since all the material of a single organism was centrifuged at the same time, comparative data would seem valid.

Growth studies were based on increase in colony size on Sabouraud glucose-agar plates and on total dry matter obtained by filtration through alundum crucibles (RA360). Colony size yielded very little quantitative data since the thickness of the colony varied and was not easily measured. Since some of the organisms may be pathogenic in spite of their long cultivation on laboratory media, they were killed by heating to boiling for three minutes, then transferred to centrifuge tubes, and centrifuged. Most of the supernatant liquid was decanted and the organisms washed thrice with distilled water to remove the culture medium. It is conceivable that some of the dry weight was lost by this method due to death and exosmosis. Where the filtrate was cloudy it was filtered again through the same crucible, resulting in a clear filtrate. Here, too, some of the dry matter may have been lost, but since the data presented should be considered as comparative and preliminary, these losses seem relatively unimportant. In the more recent data accumulated for *Saccharomyces,* boiling was omitted and a crucible (RA84) with finer pores was used.

In the preparation of media, about 100 mg. of methyl cholanthrene were shaken in 1000 ml. of distilled water and allowed to stand for three days or more, then the undissolved crystals were filtered off, and the solution here described as saturated was used to make up the various dilutions reported. All media were sterilized at 15 pounds pressure for 15 minutes and all cultures were incubated at approximately 20° C. The agar cultures were inoculated with a single loopful of a suspension of the growth of the organism on an agar slant in 5 ml. of sterile distilled water. The liquid cultures were inoculated with three drops of a suspension of organisms in a peptone glucose liquid culture from a sterile 1 ml. Ostvald pipette. The same suspension was used for all the cultures of a single series.

MORPHOLOGY

Since the differences were more pronounced in the cultures of *Saccharomyces ellipsoideus*, they will be described in detail. The other organisms showed some differences but are more difficult of interpretation and need further study.

In liquid cultures, there is a fine white sediment and no pellicle. The medium remains clear. The cells are mostly short-ellipsoid, about 5.5 μ in longer diameter. The young cells stain deeply with Maneval's stain. A deeply staining body in the center, probably the nucleus, is surrounded by a clearer zone of cytoplasm which is denser next the wall. The cells and cell groups are surrounded by a gelified sheath twice or thrice the thickness of the wall. This sheath is so transparent that it is easily overlooked, but shows more distinctly with polarized light. Protoplasmic connections persist for some time between cells. Budding may be polar, resulting in single chains of cells, but is commonly from 3 to 6 points around a great circle, resulting in a dichotomously branched filament with three points or a large plate with six points. Some of these plates suggest conditions seen in the Chlorophyceae in the Volvocales and Chlorococcales. Nuclear division is amitotic. Occasionally long slender cells, about 3 × 8.5 μ, containing about four deeply staining granules, are seen. In the older long cells, the protoplasm has contracted to the ends and the middle, leaving two ellipsoidal clear areas. Asci were not observed. In 1/40 saturation with methyl cholanthrene, the cells are similar in size and shape, the metachromatic granules are larger and sometimes fuse as small threads. In 1/10 saturation, the cells are smaller, 3–4 μ, rarely up to 5.5 μ, the cell groups contain fewer cells, the sheath is thinner, and the metachromatic granules fewer. In 1/4 saturation, cells 3–4 μ, groups rare, containing only 3–4 cells, most cells isolated, sheath thin. There are very few metachromatic granules, the vacuoles are large, the protoplast is usually next the cell wall, with a few strands crossing the vacuole. The long ellipsoidal cells are more abundant. In saturated methyl cholanthrene, the cells are larger, mostly

5.5 μ, with some 7 μ, the nucleus is central, staining deeply, the metachromatin is abundant, with thick fibres connecting the granules. The gelified sheath is very thick, and protoplasmic connections are clearly visible. Most of the cells are short-ellipsoid, a few are long-ellipsoid.

On Sabouraud glucose agar, the colony is 6 mm. in diameter, smooth, shining, nearly white and finely merulioid in the center. The cells are nearly spherical, and stain deeply. The sheath is very thin, so that the cells are mostly washed away and the arrangement is not clearly visible. The cells penetrate a short distance into the agar. The mature cells are 5.6–6 × 2–3 μ, and the nucleus is rather large and very deeply stained in the center of the cell. Budding is from two points at the distal end of the cell, resulting in dichotomously branched filaments. Rarely multipolar budding results in a whorl of blastospores at the distal end such as one commonly sees in *Syringospora*. There are usually two vacuoles at either end of the cell.

On Sabouraud agar with 1/50 saturation with methyl cholanthrene, the colony is 5 mm. in diameter, ivory-yellow, the surface smooth and dull. The cells vary from 2.5 to 3.5 μ in diameter, with many shadow cells and some larger, deeply staining cells 4–4.5 μ, spherical or nearly so. The colonies penetrating the agar are 70 × 20 μ, fusiform to ellipsoid, the outer cells deeply staining, 3–3.5 μ in diameter. Some of the deeper cells are suggestive of those seen in 1/6 saturation but are very lightly stained and difficult to see. The cells appear to be in radiating, little-branched chains.

On Sabouraud agar with 1/6 saturation with methyl cholanthrene, the colony is 8 mm. in diameter, marguerite-yellow, smooth and flat. The colony is composed of lightly staining filaments, closely dichotomously branched, ending in unbranched chains of thick-walled, deeply staining cells, suggestive of the chlamydospores of *Syringospora*. The colonies penetrating the agar are fusiform to irregular. The central tissue is composed of more or less parallel, thin-walled hyphae, lightly staining, 1.3–1.5 μ in diameter, dichotomously branched

near the tips to produce a palisade of heavily staining hyphae 6–8 cells long, twice or thrice dichotomously branched (pl. 34, figs. 1, 5). The cells are smaller with each branching, about twice as long as broad, suggesting paraphyses or the cortex of some lichens (pl. 34, figs. 2, 3). In this tissue are long ellipsoid cells which do not reach the outer surface of the colony, about $20 \times 7\ \mu$ (pl. 34, figs. 2, 6). Apparently these gradually expand into subspherical cells. At first the cells are deeply stained throughout. In somewhat older cells, the deeply staining portion is composed of a reticulum of thick fibres and small meshes, suggesting the appearance of a reticulate chloroplast. In the still older, subspherical cells (pl. 34, figs. 3, 4), the reticulum is more irregular and the meshes much larger in relation to the fibres. The whole colony is imbedded in a gel which extends about $2\ \mu$ beyond the tips of the palisade layer (pl. 34, fig. 5). In small, young colonies, the individual sheaths of the palisade hyphae can sometimes be seen within the colony gel.

FERMENTATION

Preliminary experiments using fermentation tubes show more rapid fermentation in the presence of methyl cholanthrene, producing about one-third more gas in a given time. Half saturation produces about one-fifth more gas than the control. The effects of the smaller concentrations tried are slight or doubtful. Further work with more refined technique is being carried on.

GROWTH

Colony diameter was recorded on solid media but is not a very accurate measure of growth, owing to differences in thickness in *Saccharomyces ellipsoideus* and *Mycocandida onychophila*. It seemed more reliable with the other organisms studied, but data on these organisms will be published later.

Dry-weight determinations were slower but gave much more accurate data. In our series of experiments saturated methyl cholanthrene in peptone-glucose solution has yielded 83.7 ± 1.2 mg. dry weight, while the controls under the same environ-

mental conditions yielded 64.0 ± 2.7 mg. While our data are not yet conclusive, concentrations as low as ¾ saturation yield amounts approximating that of the saturated, and those of ½ saturation or lower approach values for water. Further work is in progress to ascertain more closely the curve for total dry weight and the rate of growth at the various concentrations. The maximum variation of any cultures from the average of those at a given concentration has been 4.2 per cent, and in most cases has been much less. More variability has occurred in the lower concentrations of methyl cholanthrene.

SUMMARY

Methyl cholanthrene, the most potent of the carcinogens so far reported, profoundly affects the morphology and growth of yeasts. Giant cells and increased differentiation of cells within the colony occur in cultures of *Saccharomyces ellipsoideus* Hansen, champagne strain, after two months. Total dry weight and fermentation are increased approximately one-third in saturated methyl cholanthrene peptone-glucose solution.

EXPLANATION OF PLATE

PLATE 34

Showing the effect of 1/6 saturation with methyl cholanthrene in Sabouraud glucose agar on *Saccharomyces ellipsoideus* Hansen, champagne strain.

Fig. 1. Showing some hyphae of the central tissue and a large ellipsoidal cell in the palisade layer (× 770).

Fig. 2. Section of palisade layer, showing dichotomous branching and ellipsoidal cells dividing at their tips. The line of the surrounding gel is out of focus (× 770).

Fig. 3. Section of colony showing palisade layer, ellipsoidal cells, central tissue with large subspherical cells (× 430).

Fig. 4. Large subspherical cells (× 770).

Fig. 5. Tip of fusiform colony showing central tissue, palisade layer, and one ellipsoidal cell (× 770).

Fig. 6. Ellipsoidal cell dividing, and heavily stained subspherical cells (× 770)